Published in final edited form as:
Mol Ecol. 2009 August ; 18(15): 3256–3267. doi:10.1111/j.1365-294X.2009.04217.x.

A hybrid zone revisited: molecular and morphological analysis of the maintenance, movement, and evolution of a Great Plains avian (Cardinalidae: *Pheucticus*) hybrid zone

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Abstract

Black-headed Grosbeaks (*Pheucticus melanocephalus*) and Rose-breasted Grosbeaks (*Pheucticus ludovicianus*) are passerine bird species known to hybridize in the Great Plains of North America. Both extrinsic (environmental) and intrinsic factors (pre- and postzygotic reproductive isolation) have been credited for the generation and maintenance of the grosbeak hybrid zone, but little is known about the genetic characteristics of this hybrid zone. To investigate the stability and extent of the grosbeak hybrid zone, we constructed clines from both molecular sequence data (mtDNA, 3 autosomal intron loci, and 1 Z-linked locus) and morphological data (morphometric analyses and hybrid index scores) to determined zone center and width. Hybrid zone center and width were also determined for samples collected across the zone 40 years ago from morphological data. The present and past clines were compared and provided support for stability in hybrid zone location and width, and the evolutionary implications of this are discussed. Three models of hybrid zone maintenance were investigated to consider the influence of intrinsic and extrinsic factors on this zone. Our results suggest low hybrid frequencies, a stable zone location and narrow width, and reduced hybrid fitness over the past 40 years best categorize the grosbeak hybrid zone as a tension zone.

Keywords

Pheucticus; hybrid zone; Great Plains; mitochondrial DNA; nuclear loci; tension zone

Introduction

Secondary contact with hybridization between species is inconsistent with the classical theory of speciation, in which species are reproductively isolated from other species (Dobzhansky 1937, Mayr 1942). A hybrid zone is the area where species overlap in range and interbreed, producing viable hybrid offspring of mixed ancestry. Reproductive isolation is maintained between different species to prevent interspecific mating, but hybrid zones indicate a breakdown of premating and/or postmating barriers, often resulting in gene exchange between species and introgression of foreign alleles into pure populations. Studying the differences and frequencies in genotypic and phenotypic characteristics

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extending across the geographic distribution of zones of hybridization can help elucidate the stability and width of hybrid zones, as well as the strength of isolating barriers.

Three alternative hypotheses have been proposed to explain the maintenance of a hybrid zone. First, the bounded hybrid superiority hypothesis predicts hybrid zones to occur in intermediate “ecotones” at the interface of species boundaries, and predicts higher fitness in hybrids than parental types (Moore 1977). Second, the ecological gradient hypothesis predicts hybrid zones to occur in ecologically transitional areas that represent environmental gradients, but hybrids are conversely expected to have reduced fitness compared to parents (Endler 1977). The third model, the dynamic equilibrium hypothesis, predicts recombinant hybrid genotypes to be less fit than their parents, forming a “tension zone,” which is maintained by a balance between dispersal into the hybrid zone and selection against hybrids (Key 1968, Barton and Hewitt 1985, Barton 2001). Distinguishing between extrinsic and intrinsic selection in naturally hybridizing populations provides unique insights into the evolution of reproductive isolation and how past and present selection pressures influence species boundaries and the speciation process, as well as the causes of hybrid zone movement. Hybrid zone movement can be caused by differences in fitness, population structure, gene frequencies, and ecological differentiation throughout the zone (Barton and Hewitt 1985, Secondi et al. 2006, Buggs 2007).

Extrinsic selection involves the adaptation of organisms to the environment and the ecological interactions of organisms with their environments. In North America, many ancestral species were presumed to have diverged in response to Pleistocene climatic changes and later came into contact in areas called “suture zones,” or predicted hotspots for hybrid zone occurrence (Remington 1968). Recent studies have confirmed the existence many North American suture zones (Swenson and Howard 2004, 2005), including the Great Plains – Rocky Mountain suture zone where numerous avian hybrid zones are known to occur, representing contact between western- and eastern-distributed species (Remington 1968, Rising 1983, Price 2008). Exogenous environmental factors, such as temperature and precipitation (Rising 1970, Moore 1977, Swenson 2006) as well as anthropogenic habitat modification (Sibley and Short 1964, Short 1965, Anderson 1971), have been proposed as major factors responsible for past and present maintenance of avian hybrid zones in this suture zone. In contrast, endogenous selection reflects within genome or organism effects and is independent of the environment, which may result in intrinsic biological barriers to mating and reduced fitness of hybrid offspring. Sexual selection based on plumage, morphology, or song can be an effective intrinsic mechanism for premating reproductive isolation in birds (Grant and Grant 1997), preventing interspecific mating from occurring. Intrinsic premating isolating mechanisms also include mating incompatibilities and fertilization (syngamy) failure (Birkhead and Brillard 2007). If hybrid offspring are successfully produced, postmating isolating mechanisms, including sexual and ecological selection against hybrids and genetic incompatibilities, may result in reduced fitness in hybrids. Hybrid offspring are often predicted to have lower fitness than parental types and fitness disadvantages of hybrids include sterility, reduced viability, reduced fertility, and/or F_1 hybrid behavioral difficulties. Haldane's Rule (1922) predicts the heterogametic sex of hybrid offspring (avian females) will exhibit reduced fitness compared to the homogametic sex and has been supported in studies of avian hybridization (Price and Bouvier 2002).

This study characterizes the zone of hybridization between two grosbeak (*Pheucticus*) species in South Dakota, U.S.A. using molecular and morphological methods. The Black-headed Grosbeak *(Pheucticus melanocephalus)* occupies a western breeding range (Hill 1995), while the Rose-breasted Grosbeak *(Pheucticus ludovicianus*) occupies a northeastern breeding range in North America (Wyatt and Francis 2002). These two woodland species hybridize along riparian corridors where their ranges overlap in the Great Plains – Rocky Mountain suture zone. In the Great Plains, interspecific *Pheucticus* hybridization has been reported to be most extensive in Nebraska (West 1962, Rising 1983), but less common in the Dakotas due to habitat availability (Anderson and Daugherty 1974, Kroodsma 1974b). In eastern South Dakota, a gap in the zone of hybridization exists due to the lack of streams and rivers spanning eastward from the Missouri River, leaving little suitable grosbeak habitat. *Pheucticus ludovicianus* is common in the southeastern portion of South Dakota and hybridization with *P. melanocephalus* does occur along the Missouri River (West 1962, Rising 1983).

The two *Pheucticus* species are distinguished primarily by morphology. Male *P. melanocephalus* individuals are identified by the presence of a yellow breast, belly and underwing color and brown underparts, rump, hind, and other areas; male *P. ludovicianus* are identified by a contrasting rose-red breast, belly and underwing color and an absence of the brown coloration, replaced by white (Anderson and Daugherty 1974). Females of the two species, on the other hand, are morphologically similar and more difficult to distinguish, differing in underwing color and extent of streaking and color of the breast and belly (West 1962). Interspecific hybrid offspring may be morphologically similar to either parent species, or reflect any range of phenotypes intermediate to the parent species (West 1962, Anderson and Daugherty 1974, Kroodsma 1974b).

Past studies on *Pheucticus* hybridization in the Great Plains have used morphological plumage differences between parent species and hybrids to distinguish between pure and hybrid individuals (West 1962, Anderson and Daugherty 1974, Kroodsma 1974b). Premating and postmating reproductive isolation have both been hypothesized to be important in the maintenance of the Great Plains *Pheucticus* hybrid zone (West 1962, Anderson and Daugherty 1974, Kroodsma 1974a, Kroodsma 1974b). Anderson and Daugherty (1974) found some evidence of both female assortative mating and reduced viability of hybrid females within the *Pheucticus* hybrid zone. Further molecular analysis of this hybrid zone should provide additional insight into the structure, maintenance, and stability of this hybrid zone.

This study morphologically and genetically characterized the contemporary *P. melanocephalus* x *P. ludovicianus* hybrid zone in South Dakota to determine the current center and width of this zone. We additionally reevaluated historical morphological data on this hybrid zone to determine the stability and location of this zone over the past 40 years, which further allowed us to investigate models of hybrid zone maintenance. Three hypotheses were evaluated: 1) the grosbeak hybrid zone was predicted to be stable in its location in South Dakota and of similar width to that described by Anderson and Daugherty (1974) during the 1960's, 2) hybridization between these two species was predicted to be infrequent and hybrid individuals rare in the hybrid zone, and 3) extrinsic and intrinsic

factors both were predicted to be responsible for the maintenance of the grosbeak hybrid zone. This was done by exploring patterns of morphological (hybrid index scores and morphometric measurements) and genetic (maternal mitochondrial DNA and biparental autosomal nuclear DNA and Z-linked DNA) differentiation across the grosbeak hybrid zone and investigating clinal variation in these characters.

Methods

Samples

Individuals were located and identified in the field by call playback. We collected 143 individuals during the 2007 breeding season, comprised of 140 (133 males and 7 females) adult birds and three eggs. Individuals were collected in a west-to-east transect across South Dakota in riparian areas and gallery forests along Spearfish Creek, White River, Bad River, Missouri River, and Big Sioux River and individuals were grouped into ten sampling localities based on longitude for further analyses (Figure 1). The western extent of the collecting localities was near Spearfish, SD (Locality 1) historically representing pure *P. melanocephalus*, while the eastern extent was Newton Hills State Park near Canton, SD (Locality 10), representing pure *P. ludovicianus*. Latitude, longitude, and altitude of each individual's locality were recorded using Global Positioning System (GPS). Study skins were prepared from each specimen and deposited into collections at Black Hills State University (Spearfish, South Dakota).

Morphology and Plumage Measurements

The mass of each adult was measured in the field and four morphological measurements were made in the lab including: bill length (from anterior edge of nare to bill tip), bill width (at its base), wing cord (flattened wing from bend of wing to longest primary), and tarsus length (from intertarsal joint to distal end of last leg scale). Individuals were identified as pure individuals or hybrids using the same hybrid index scoring criteria of Anderson and Daugherty (1974) based on the coloration of five plumage characters for males (nape, flank, back, rump, and breast) and three characters for females (throat/breast/side of neck, extent of yellow, and extent of streaking). From these characters, each male individual was categorized as one of the following five phenotypes: pure *P. melanocephalus*, other hybrid more similar to *P. melanocephalus*, intermediate hybrid, other hybrid more similar to *P. ludovicianus*, or pure *P. ludovicianus* according to Anderson and Daugherty (1974). Yearling males were combined with adult males to be consistent with the scoring methods of Anderson and Daugherty (1974), who indicated subadult and adult males were found to have nearly identical hybrid index scores. Females were classified similarly, but excluded from further morphological analyses due to low sample sizes and highly variable morphologies. Principal Components Analysis (PCA, SYSTAT ver 12) was used to analyze bill length, bill width, wing cord, and tarsus length of intact male specimens (N=129).

Genetic Data Collection

Breast muscle or embryonic tissue (from eggs) was obtained from each individual, placed in a 2 mL cryotube, and stored in liquid nitrogen in the field. Upon return to the laboratory, samples were stored at −80°C prior to DNA extraction. Total genomic DNA was extracted

using either a DNeasy Tissue Kit (Qiagen, Valencia, CA) or phenol:chloroform:isoamyl alcohol with Phase Lock Gel Light (Eppendorf) at the Western South Dakota DNA Core Facility (WestCore) at Black Hills State University. Total genomic DNA was quantified with a NanoDrop spectrophotometer (Thermo Scientific) and each sample was diluted to 60 ng/μl for optimal PCR amplification and stored at −20°C.

Preliminary PCR amplification and sequencing was performed at the mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 2 (mt-ND2) gene using the primers L5215 (Hackett 1996) and H6313 (Johnson and Sorenson 1998). We designed internal primers L325 and H565 from these sequences to target a short fragment of the ND2 gene approximately 200 bp in length that was diagnostic between the two species. This ND2 fragment was then PCR amplified with the primers L325 and H565 in all 143 *Pheucticus* samples obtained in 2007 from South Dakota. Four unlinked nuclear loci mapped to intron regions of the chicken genome (*Gallus gallus*) were amplified in the 143 *Pheucticus* samples and include: PCBD1 (Pterin-4 alpha-carbinolamine dehydratase), intron 2; FGB (Beta-fibrinogen), intron 5; RHO (Rhodopsin) intron 2; and MUSK (muscle skeletal tyrosine kinase), intron 4 and Z-linked (Table S1). These four nuclear loci and the mitochondrial gene were PCR amplified in 15 μl reactions using either 2X Promega Master Mix or Eppendorf 2.5x HotMasterMix with a GeneAmp 9700 PCR System (Applied Biosystems) under the following conditions: 94°C denaturation for 10 min, followed by 35 cycles of 94°C for 30 s, 54 – 56°C for 30 s (see Table S1 for annealing temperatures), 72°C for 1 min, followed by a 72°C extension for 7 min, and a 4°C hold.

Amplified products were purified using ExoSAP-IT (USB Corporation) following the manufacturer's protocol. BigDye® Terminator v3.1 (Applied Biosystems) cycle sequencing reactions using 20–40 ng of purified PCR product were run on a 9800 Fast Thermal Cycler (Applied Biosystems) under the following conditions: 96°C denaturation for 1 min, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 1 min and 15 s, and followed by a 4°C hold. Cycle sequencing reactions were purified with the Agencourt CleanSEQ Dye Terminator Removal kit and run on an Applied Biosystems 3130 Genetic Analyzer at WestCore. Complementary strands at the mt-ND2 locus and the four nuDNA loci were aligned by eye using SEQUENCHER ver 4.8 (Gene Codes Corporation, Ann Arbor, MI). Ambiguous nuclear SNP loci were identified visually in SEQUENCHER with the presence of two peaks of equal intensity and assigned the appropriate IUPAC ambiguity symbols. Sequences of unique haplotypes at the five loci were submitted to GenBank with the following accession numbers: PCBD1 (*N*=13), FJ004624-FJ004636; FGB (*N*=86), FJ010364-FJ010449; RHO (*N*=13), EU934822-EU934834; MUSK (*N*= 98), FJ010450-FJ010546; and mt-ND2 (*N*=2), FJ040875-FJ040876.

Data Analysis

Assignment of mt-ND2 haplotypes to either parental species was performed via maximum parsimony and maximum likelihood phylogenetic analysis in PAUP* ver 4.10b (Swofford 2001). Nuclear sequences were initially analyzed with PHASE ver 2.1 (Stephens et al. 2001, Stephens and Scheet 2005) to infer phase and reconstruct the haplotypes of each individual. A Bayesian analytical framework and Markov chain- Monte Carlo (MCMC) algorithm were

used for this analysis and haplotypes were inferred at the 95% confidence level. The hybrid model in PHASE ver 2.1 was used for phase reconstruction, which implements the faster original model for preliminary computations and the newer recombination model for final computations (Stephens et al. 2001, Stephens and Scheet 2005).

TOPALi ver 2 software (Milne et al. 2004) was used to detect the presence of recombination at the four nuclear loci using the Difference of Sums of Squares (*DSS*) method and implementing a sliding window of 100 bp and 10 bp step size at a 95% confidence level. Due to the large number of sequences, TOPALi calculated a random selection of 50 sequences for each of two runs. TOPALi was additionally used to evaluate the fit of 56 models of sequence evolution to each locus. The Jukes Cantor model of nucleotide substitution was used to initiate analysis at each locus and Akaike information criterion (AIC1) scores were used to determine the substitution model with the highest likelihood, while minimizing the number of parameters.

We quantified sequence polymorphism at each nuclear locus using DnaSP ver 4.20.2 software (Rozas et. al 2003) and the following statistics: number of haplotypes, h; haplotype diversity, Hd (Nei 1987); nucleotide diversity, π (Nei 1987); average number of nucleotide differences, K (Tajima 1983); and θ ($4N\mu$) per sequence and per site (Nei 1987, Tajima 1993). Departures from neutrality at each locus were tested with Tajima's (1989) and Fu and Li's (1993) tests. Network ver 4.500 (Bandelt et al. 1999, www.fluxus-engineering.com) was used to reconstruct networks and visualize relationships among haplotypes at each nuclear locus using the median-joining (MJ) network algorithm. Default values of epsilon = 0 and weighting transversions/transitions 1/1 were used. Length polymorphisms and/or low confidence in phase reconstruction resulted in only 138 (of 143) individuals being used for analyses of nuclear loci. All polymorphic sites (123 total sites) found in the complete 4 locus dataset (138 individuals) were converted to four state SNP data using a python script (Nexus2Structure.py; Dr. Charles Chapuis, pers.comm.).

Cline Construction

The distance across the collecting transect (encompassing the hybrid zone) was measured using ArcGIS 9/ArcMap ver 9.2 (ESRI) as a linear distance along river corridors from western to eastern South Dakota. Measurement of distance began in the Black Hills in western SD (Locality 1) as 0 km and ended in eastern SD near Canton, SD (Locality 10) at 636 km. Maximum likelihood (ML) clines were fitted to morphological and genetic data using the software package ANALYZE (Barton and Baird 1996), which implements the tanh cline model of Szymura and Barton (1986). The tanh cline is defined as $y = (1 + \tanh(2(x-c)/w))/2$, in which x is the distance from the center of the cline, c is the center of the cline, and w is the width of the cline, calculated as 1/maximum slope (Szymura and Barton 1986). Population averages at all ten localities were used to fit clines for 2007 male hybrid score and mtDNA, while samples at Locality 2 (202 km) were combined with Locality 3 (242 km) for PC1, RHO and PCBD1 clines due to outliers in the data and small samples size at Locality 2. All data sets were scaled to p_{min} and p_{max} of zero and one, respectively and a simple single locus was assumed for each run to make the program amendable to morphological and quantitative data. ANALYZE used the Metropolis-Hastings algorithm

(Metropolis et al. 1953, Hastings 1970) to estimate *c* and *w* from multiple runs on population (locality) averages of the 2007 data, including: hybrid index scores, PC1, PCBD1 haplotype frequencies, RHO haplotype frequencies, and mtDNA frequencies. Several runs were implemented for each character, and the best run and overall support was estimated with log-likelihood scores, representing unconstrained ML values.

Additionally, hybrid index scores were reevaluated from Anderson and Daugherty's (1974) historical samples from South Dakota and Nebraska, fit to our transect scale, and ANALYZE was used to estimate *c* and *w* to directly compare with the contemporary data from this study. Sigmoid clines were graphed using the tanh equation for contemporary and historical characters using the Mathematica ver 6 (Wolfram) statistical package.

To further explore cline center coincidence and cline width concordance, likelihood-ratio tests, as described by Hilborn and Mangel (1997), were first performed between contemporary characters by exploring simple (Model A) vs. more complex (Model B) data sets. The null hypothesis for each test was no significant difference between the two models. Additional Metropolis-Hastings searches were implemented in ANALYZE by constraining the parameters (*c* or *w*) to Models A or B estimating log-likelihood scores for all five characters. The test statistic, *R*, was calculated as the absolute difference between the sum of likelihoods for Model A and Model B, multiplied by two. The significance of *R* was determined from a chi-square table with the degrees of freedom equal to the number of characters minus one.

Additional likelihood-ratio tests were used to determine concordance between the clines constructed for past and present hybrid index scores. The null hypothesis for these tests was no difference between past and present *c* and *w*. ANALYZE determined new log-likelihoods by constraining *c* or *w* to the average between past and present cline data. *R* was similarly calculated as the absolute difference between the sum of the new likelihoods and the sum of the original likelihoods, multiplied by two. Significance was determined by chi-square goodness-of-fit.

Results

Morphology

Using Anderson and Daugherty's (1974) hybrid index scoring system based on plumage attributes, we classified the 129 male individuals collected in 2007 as follows: 80 pure *P. melanocephalus*, five other hybrids more similar to *P. melanocephalus*, six intermediate hybrids, eight other hybrids more similar to *P. ludovicianus*, and 30 pure *P. ludovicianus.* Morphologically pure *Pheucticus* were collected in localities 1 – 5, while morphological hybrids were collected in localities 6 – 10 (Figure 1 and Table 1). Seven total females were collected including one pure *P. melanocephalus*, one other hybrid more similar to *P. melanocephalus*, four individuals more similar to *P. ludovicianus*, and one pure *P. ludovicianus*.

The PCA analysis of the morphological measure determined that the first principal component axis (PC1) explained most of the variation in morphological measurements

(9.259%), which explained 84.288% of the total variation. The highest component loading for PC1 (3.005) was wing cord. PC1 exhibited clinal variation, with higher population averages at locality one and lowest averages at locality ten (Figure 2), allowing use of PC1 for further cline analysis. The second principal component (PC2) explained had highest loadings for bill width, bill length, and tarsus (0.081–1.003). PC2 was not clinal across the sampling landscape (Figure 2), and explained only 9.869% of the total variation in morphological measurements, therefore PC2 was not used in further analyses.

Mitochondrial DNA

Phylogenetic analysis of the mt-ND2 gene identified two well-supported mitochondrial clades corresponding to the two *Pheucticus* species. A total of 96 individuals were assigned to the *P. melanocephalus* clade and the number of birds morphologically identified as *P. melanocephalus*, *P. ludovicianus*, and *Pheucticus* hybrids in this clade were 92, 0, and 4 respectively. A total of 50 individuals were assigned to the *P. ludovicianus* clade and the number of individuals morphologically identified as *P. melanocephalus*, *P. ludovicianus*, and *Pheucticus* hybrids in this clade were 1, 42, and 7 respectively. The single *P. melanocephalus* male (from locality 6) that was found to have *P. ludovicianus* mtDNA had primarily *P. melanocephalus* morphology, with only slight (if any) indication of *P. ludovicianus* characteristics; this individual was therefore most likely of mixed ancestry, but displayed a *P. melanocephalus* phenotype. Mixed proportions of *P. melanocephalus* and *P. ludovicianus* mtDNA were detected in the region spanning from 100.0° to 99.0° W. Mitochondrial DNA haplotype frequencies at each locality were used to construct a contemporary mtDNA cline (see Cline Analysis).

Nuclear DNA

PHASE reconstructed the best haplotype estimates for each individual at the four nuclear loci as follows: 13 haplotypes (10 variable sites) at PCBD1 with 450 bp of sequence, 86 haplotypes (53 variable sites) at FGB with 574 bp of sequence, 13 haplotypes (8 variable sites) at RHO with 287 bp of sequence, and 98 haplotypes (53 variable sites) at MUSK with 392 bp of sequence. Both PCBD1 and RHO networks show strong relationships and distinguish *P. melanocephalus* and *P. ludovicianus* haplotypes relatively well (Figure S1), which supports the geographic distribution of the two *Pheucticus* species in South Dakota, therefore haplotype frequencies at these two loci were used to construct nuclear DNA clines. FGB and MUSK contained considerably more variation compared to PCBD1 and RHO, revealing more shared haplotypes between the parental species, therefore excluding FGB and MUSK from cline analyses. Neutrality tests of Tajima (D), Fu and Li (D* and F*) test statistics were not significant at the four nuclear loci. Sequence polymorphism statistics determined by DNAsp for each of the four nuclear loci are shown in Table 2. Overall, greater variation and diversity were found at the MUSK and FGB loci compared to the RHO and PCBD1 loci.

TOPALi analyses found no evidence of significant recombination at any of the four nuclear loci; all *DSS* values were determined to be lower than the 95% significance point for both runs at each locus and supported the null hypothesis of no recombination. The best-fit model of substitution, rate heterogeneity (Γ), proportion on invariant sites (pINV), likelihood of

model ($-\ell$), Akaike information criterion (AIC1), AIC with second order correction (AIC2), and proportion of transitions to transversions (Ts/Tv) for each locus are listed in Table S2.

Cline Analysis

Although *DSS* values determined no recombination at the four nuclear loci, it appears that recombination may be present in MUSK and FGB from the structure of the networks at these loci (Figure S1), therefore these two loci were subsequently excluded from cline analysis. Cline centers, widths, and log likelihood scores are listed in Table 3 for the five contemporary clines including: 2007 male hybrid index scores, PC1, nuDNA PCBD1, nuDNA RHO, and mtDNA ND2, as well as historical hybrid index scores. Visual inspection of the contemporary clines (Figure 3a) revealed three relatively concordant clines (male hybrid score, mtDNA, and PCBD1) with steeper slopes; these three clines had similarly coincident center (c) estimates and were considered together as Model A in subsequent analyses, constraining the consensus center to the average c of 459 km and simplifying the dataset. The average c of all five clines is shifted westward to 405 km by the variation represented by RHO and PC1 clines; the five clines are considered together as Model B in subsequent analyses, representing a more complex model. The likelihood-ratio test including all five contemporary characters (Model B) indicated no significant difference between c likelihoods of 405 km and 459 km ($R_{cB} = 0.838$, df = 4, $P > 0.05$). The likelihood-ratio test for Model A (three characters) determined a significant difference between c likelihoods ($R_{cA} = 27.936$, df = 2, $P < 0.05$), suggesting Model A to have a better likelihood over Model B. Therefore, the three-character model with an average c of 459 km is the most likely center estimate from the contemporary cline data.

The average width (w) likelihoods for Models A and B were determined to be 114 km and 187 km, respectively. The Model B likelihood-ratio test for w determined a significant difference between cline widths of 114 km and 187 km ($R_{wB} = 88.0$, df = 4, $P < 0.05$); Model B's average of 187 km was calculated to be the most likely estimate of contemporary cline width.

Our contemporary male hybrid index scores were compared to the historical scores of Anderson and Daugherty (1974) by estimating c and w for the historical data and comparing cline profiles. Visual inspection revealed cline centers that are relatively coincident between contemporary and historical data, 435 km and 449 km respectively (Figure 3b). New likelihoods were determined for 2007 and historical clines using ANALYZE to constrain c to the average of these two clines, 442 km. A likelihood-ratio test between the sums of new and original likelihoods determined no significant difference between the original estimates of c and the average c ($R_{cIndex} = 1.252$, df = 1, $P > 0.05$), suggesting no significant change in the center of the hybrid zone over the last 40 years.

Initial cline analysis revealed that cline widths differed more substantially (by 92 km) between past and present male hybrid index scores (Table 3). The average w for the past and present clines was 152 km, so new likelihoods were estimated for 2007 and historical clines by fixing w to this width. A likelihood-ratio test between the sums of new and original likelihoods determined a significant difference between the average w likelihoods and the

original unconstrained likelihoods (R_{wIndex} = 6.782, df = 1, $P < 0.05$). This result supports a significant reduction in the width of the hybrid zone over the last 40 years.

Discussion

Zone Movement vs. Stability

To accurately detect clinal signatures of zone displacement, concurrent trends in multiple genetic markers are needed to reliably confirm evidence of zone movement (Barton and Hewitt 1985, Buggs 2007), but this has proven to be challenging in avian hybrid zone research (Vallender 2007). Our multi-locus and morphological cline analyses suggest the best estimate of the center of the zone of grosbeak hybridization in South Dakota is currently located at 459 km along our collecting transect, corresponding to a longitude of approximately 98.8° W within sampling locality eight. Most notable perhaps it the concordance of our estimated cline center with historical estimates of the major area of hybridization. In the 1960's and 1970's, Anderson and Daugherty (1974) found the greatest frequency of morphological hybrid grosbeaks to be just northwest of Greenwood, SD (98.4° W), based on hybrid index scores. Near Greenwood, 63% of the individuals collected by Anderson and Daugherty (1974) were classified as hybrids, and our sampling in this area (Locality 9, average distance of 498 km) also yielded the highest frequency of morphological hybrids (44% intermediate + other hybrids). However, we believe hybridity may be grossly overestimated using Anderson and Daugherty's hybrid index. Differences in phenotypic and genotypic hybrid frequency reflect the importance of investigating both morphological and genotypic variation when studying hybrid zones in the present day.

We reevaluated Anderson and Daugherty's original data using cline analysis of historical hybrid index scores, and our results suggest the historical hybrid zone center was more accurately positioned at 449 km (about 99.1° W), approximately 50 km westward of the investigators' previous estimate of the major area of hybridization (with the greatest proportion of admixed individuals) near Greenwood, SD. At 99.1° W near St. Charles, SD, Anderson and Daugherty (1974) found more equal proportions of *P. ludovicianus* and *P. melanocephalus*, and therefore this location is a better estimate of the midpoint between the grosbeak species and the historical hybrid zone center. Like Anderson and Daugherty (1974), we combined subadult and adult males in our analyses of contemporary Grobeak hybridity to be consistent with historical sampling in South Dakota and reduce scoring bias between the two studies. Although we are confident subadult and adult males were accurately scored using this method; applying further morphological age class corrections to both historical and contemporary males may be a better estimate of hybridity in this zone, but was not possible at this time.

Our tests further suggest no significant difference between historical and contemporary cline center estimates, 449 km and 435 km respectively. Such similarity between past and present cline centers implies no significant movement of the transition point between species or the grosbeak hybrid zone in South Dakota over the past 40 years, providing overall support for the stability of the Grosbeak hybrid zone during this time span. Additionally, limited biparental gene flow between grosbeak species (relatively narrow nuclear DNA clines) and low maternal gene flow (narrow mtDNA cline) across the zone of hybridization suggests the

maintenance of species boundaries. Interestingly, the first record of *P. melanocephalus* and *P. ludovicianus* contact in South Dakota was reported in 1856 approximately just 6 km east of the historical cline center at 99.1° W (Baird 1858) and hybridization was first documented in 1937 about 25 km west of 99.1° W (West 1962); this data further suggests the grosbeak hybrid zone may have remained stable in its location for an even longer time period, perhaps over the past 150 years.

Next we considered the variation in zone widths among contemporary characters, ranging from 82–356 km wide (Table 3). The most likely contemporary cline width considering all five characters was determined by our tests to be 187 km, the average width of the five clines (Model B). Past and present estimates of hybrid zone width determined from the clines obtained from the historical and contemporary hybrid index score data differed substantially, 198 km and 106 km respectively, and were determined to be significantly different; this may initially suggest that the width of the grosbeak hybrid zone has narrowed approximately 90 km over the past 40 years. Alternatively, the relatively narrow single cline width estimated for the contemporary hybrid index cline may be underestimated due to the smaller sample size of individuals across the hybrid zone (this study N = 128 males, Anderson and Daugherty, 1974, N = 363males). Likewise, the single historical hybrid index cline width may be overestimated due to Anderson and Daugherty's likely over-count of historical hybrids. This inconsistency utilizing a single character demonstrates the importance of considering both morphological and genetic characteristics of modern hybrid zones. We suggest the five-cline contemporary cline width average of 187 km is a more accurate estimate of the current zone width because it incorporates both genetic (nuDNA and mtDNA) data and morphological data (hybrid index and PC1) to best characterize hybrid zone width; this estimate is remarkably similar to the historical hybrid index cline width estimate of 198 km, which would again offer support to the overall stability of the grosbeak hybrid zone over the past 40 years.

Evaluating Hybrid Zone Models

It has been argued that extrinsic selection has played a primary role in the formation and maintenance of avian hybrid zones in the Rocky Mountain – Great Plains suture zone through the past and present influence of temperature, precipitation, and aspect in this region (Swenson 2006). Sibley and Short (1959) suggested the extrinsic influence of climate change and man's tree planting created suitable habitat in the Great Plains allowing contact between another hybridizing passerine species pair, the Indigo Bunting (*Passerina cyanea*) and Lazuli Bunting (*P. amoena*). Habitat isolation has also been proposed to be maintaining boundaries between two hybridizing species of towhees (*Pipilo erythrophthalmus* and *P. maculatus)* in the Great Plains (Sibley and West 1959). It was postulated that grosbeak dispersal has been significantly limited by the lack of suitable habitat east of the Missouri River (Rising 1983) and by habitat destruction resulting from reservoir construction along the Missouri River (Anderson 1971, Anderson and Daugherty 1974, Kroodsma 1974). Anderson and Daugherty neglected to collect along Lake Francis Case in the 1960's, which was likely due to the transition and inundation of woodland habitat along the Missouri River following the construction of Fort Randal Dam and Francis Case Reservoir in 1956; alternatively, Anderson and Daugherty successfully collected grosbeaks south of the

reservoir, along Ponca Creek. In 2007, we conversely found the greatest frequency of heterospecifics and hybrids along Lake Francis Case; this demonstrates, that although habitat characteristics within the grosbeak hybrid zone have changed significantly over the past 40 years, grosbeak hybridization is still occurring (although not commonly) in the same location in South Dakota. Therefore, extrinsic environmental factors may have had little influence on the stability of the grosbeak hybrid zone over time.

Alternatively, we suggest intrinsic endogenous factors, the interaction between species, may be more responsible for the maintenance of zone center and width over the past 40 years. Our cline analyses indicate the position and width of the grosbeak hybrid zone has remained stable over this time period, and this stability and narrow cline widths may suggest the intrinsic maintenance of the hybrid zone in the face of environmental transition. Anderson and Daugherty (1974) historically found a significant decrease in hybrid female clutch size compared to pure female clutch size, implying reduced female hybrid fitness and suggesting Haldane's Rule [1922, which expects the heterogametic sex (avian females) to exhibit reduced fitness prior to the homogametic sex] may be important within the zone of contact. In addition to Haldane's Rule, positive assortative mating was previously suggested to be occurring in the grosbeak hybrid zone (Anderson and Daugherty 1974, West 1962), and other passerine hybrid zones in the Great Plains (Baker and Baker 1990, Baker and Boylan 1999, Sibley and West 1959). In other studies of hybridizing avian species pairs, character displacement of male plumage traits has been shown to be sex-linked in sympatric hybridizing bird populations (Sætre et al. 2003), and can strongly influence the presence of female assortative mating in hybrid zones (Sætre et al. 1997) and contribute to zone stability and maintenance. Although, we made no direct measurements regarding clutch size or overall fitness of hybridizing pairs; the position and extent of the contemporary grosbeak hybrid zone compared to the historical zone does support Anderson and Daugherty's hypothesis of reduced hybrid fitness. Future study of genomic variation, comparison of patterns of autosomal and sex-linked loci, across the hybrid zone will provide a better understanding of the intrinsic factors maintaining zone stability.

The three classic models of hybrid zone maintenance propose differing roles for extrinsic and intrinsic selection in hybrid zones. The bounded hybrid superiority model predicts hybrid zones to occur in ecologically transitional areas and predicts a higher frequency and higher fitness of hybrids compared to parentals in intermediate environments. We suggest both past and present hybrid frequency estimates were overestimated using the hybrid index scoring methods of Anderson and Daugherty, and argue grosbeak hybrids are rare in this hybrid zone. Since there are many difficulties associated with morphological variation between these grosbeak species (and hybrids), utilizing admixture models (Buerkle and Lexer 2008) and/or historical DNA samples may aid in better estimation of both past and present hybrid frequencies. Our evidence of low hybrid frequency, narrow cline widths, and reduced hybrid fitness effectively reject hybrid superiority as a plausible model for this hybrid zone.

The ecological gradient hypothesis alternatively predicts a lower occurrence of hybrids compared to pure types along an environmental gradient, which would help explain the gradual transition in morphologies and hybrid frequencies across the zone of hybridization.

However, as previously mentioned, there have been dramatic changes in habitat composition along the Missouri River due to dam erection and flood-plain inundation since the time of Anderson and Daugherty's (1974) study. If environmental selection during such habitat transition was indeed occurring and the grosbeak species were tracking the changing environment, the ultimate result would be zone movement (change in zone center or width). We have provided evidence that the grosbeak hybrid zone has remained stable in both location and width for at least the last 40 years (and potentially for the past 150 years or more); therefore, the grosbeak hybrid zone is not likely maintained by environmental gradients (in habitat).

The general narrow width of our clines and stability of the grosbeak hybrid zone over the past 40 years in the face of dramatic environmental change indicate selection against hybrids is a primary mechanism responsible for the maintenance of this hybrid zone. Historical data additionally suggests the interactions between organisms via positive assortative mating (Anderson and Daugherty 1974, West 1962) are an important influence on the grosbeak hybrid zone. Within the hybrid zone, pure grosbeak species are at the edge of their respective distributions, and therefore dispersal into the zone is likely low. For these reasons, we suggest the grosbeak hybrid zone is best described by the dynamic equilibrium model, which proposes the zone has likely been maintained by a balance between dispersal into the zone and selection against hybrids; this qualifies the grosbeak hybrid zone best as a tension zone. Price (2008) demonstrated that hybrid zones between long-diverged taxa are often characterized by relatively narrow zone widths and low proportions of hybrids in the center of the zone, classifying these zones as tension zones. Additionally, Price (2008) suggests such tension zones may be likely to exhibit a high degree of both premating and postmating isolation, including social and ecological selection and selection against hybrids. The (mtDNA cyt b) divergence between *P. melanocephalus* and *P. ludovicianus* was previously estimated at 2.2 million years (Klicka and Zink 1997), therefore we argue the deep divergence between these species, indication of hybrid fitness reduction, and narrow width of the grosbeak hybrid zone best categorizes this zone as a tension zone according to Price.

Conclusion

Hybrid zones have been described as `natural laboratories for the study of evolutionary biology' (Hewitt 1988). As in the laboratory, the ability to study an experiment over time enhances the conclusions that can be drawn from the data. In this study, the ability to compare hybrid zone dynamics from contemporary and historical samples (separated by 40 years) provided a means of testing hypotheses about the evolution of a hybrid zone, and also provided additional support (stable location and width of the hybrid zone) for the classification of the grosbeak hybrid zone as a tension zone. Although, our ability to infer the exact evolutionary processes responsible for maintaining zone stability are beyond the reach of the data presented here; recent advances in obtaining genomic data from museum skins to compare to genomic data from the contemporary samples could certainly further elucidate the evolutionary architecture of the hybrid zone and perhaps the architecture of speciation in these two species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank John Klicka with the Marjorie Barrick Museum of Natural History and the Bell Museum of Natural History for providing tissue samples for this study. We also thank Doug Backlund and state and federal agencies for collecting permits, the numerous wildlife officers for their cooperation, committee members Cynthia M. Anderson, Shane K. Sarver, Dave H. Siemens, and Scott F. Stoltenberg for comments and advice on early drafts of this manuscript, and Jess Moser, Forrest Cain, and Shane Ziegenbein for field assistance. Additional thanks to Parthasarathi Nag for help with cline construction. Laboratory work was completed at the Center for the Conservation of Natural Resources (CCBR)/Western South Dakota DNA Core Facility (WestCore) on the campus of Black Hills State University. This project was funded in part by CCBR/EPA Grant # US EPA CR-83152201-0 and funding from Black Hills State University. The project described was also supported in part by Grant Number 2 P20 RR016479 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

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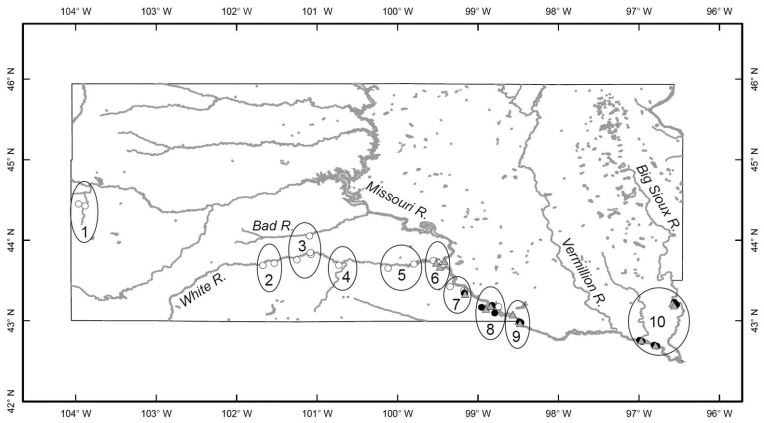

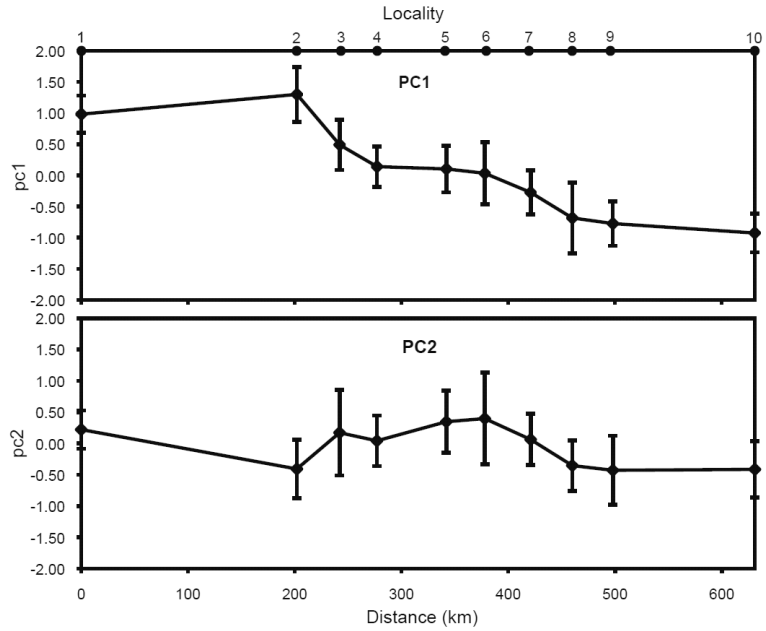

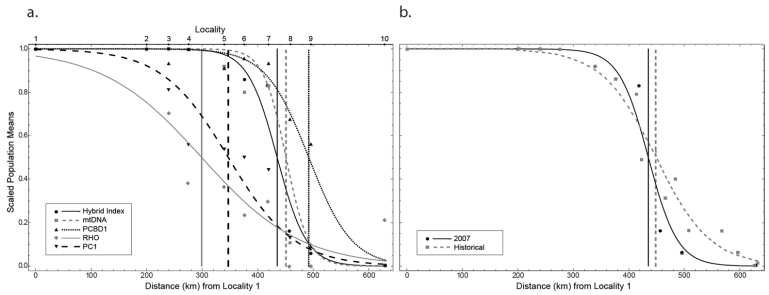

Locality (Distance, km)	*N*	Pure Pme	Other Pme	Intermediate	Other Plu	Pure Plu
1 (0)	22	22	0	0	0	0
2 (202)	3	3	0	0	0	0
3 (242)	16	16	0	0	0	0
4 (277)	12	12	0	0	0	0
5 (342)	14	13	0	0	0	1
6 (378)	10	7	1	2	0	0
7 (421)	12	6	4	1	0	1
8 (460)	9	1	0	1	2	5
9 (498)	9	0	0	1	3	5
10 (631)	22	0	0	1	3	18

locus	# seqs	# sites	S	Eta	h	Hd	Π	k	θ (per seq)	θ (per site)
MUSK	292	392	53	55	98	0.96	0.00991	3.75479	8.79682	0.02321
RHO	292	287	8	9	13	0.4	0.00188	0.45867	1.43948	0.0059
FGB	278	574	53	54	86	0.968	0.01489	7.00039	8.70541	0.01852
PCBD1	288	450	10	11	12	0.62	0.0055	2.12991	1.76326	0.00456

Character	Cline Center (c), km	Cline Width (w), km	Log Likelihood
2007 hybrid index	435	106	−2.096
mt-ND2	451	82	−0.354
PCBD1	492	155	−2.061
RHO	299	356	−6.332
PC1	347	238	−0.301
Historical hybrid index	449	198	−8.473